

**Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol**

**Supplemental Assay Method for Titration of Feline
Rhinotracheitis Virus in Cell Culture**

Date: December 16, 1998
Supersedes: SAM 307, Dated: January 14, 1983
Number: MVSAM0307.01
Standard Requirement: 9 CFR 113.315
Contact Person: Larry R. Ludemann, (515) 239-8264
Marsha J. Hegland, (515) 239-8659
Approvals:

Linn A. Wilbur, Head/Team Leader
Mammalian Virology Section
Date: _____

P. Frank Ross, Acting Quality Assurance Manager
Date: _____

_/_s/ Randall L. Levings_____
Randall L. Levings, Director
Center for Veterinary Biologics-Laboratory
Date: _12/16/98

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

Mention of trademark or proprietary product does not constitute a guarantee or warranty of the product by USDA and does not imply its approval to the exclusion of other products that may be suitable.

Supplemental Assay Method for Titration of Feline Rhinotracheitis Virus
in Cell Culture

Table of Contents

1. Introduction
2. Materials
 - 2.1 Equipment/instrumentation
 - 2.2 Reagents/supplies
3. Preparation for the test
 - 3.1 Personnel qualifications/training
 - 3.2 Preparation of equipment/instrumentation
 - 3.3 Preparation of reagents/control procedures
 - 3.4 Preparation of the sample
4. Performance of the test
5. Interpretation of the test results
6. Report of test results
7. References
8. Changes

Supplemental Assay Method for Titration of Feline Rhinotracheitis Virus
in Cell Culture

1. Introduction

This is an *in vitro* titration method for assaying modified-live feline rhinotracheitis virus (FRV) vaccines for viral content. The method uses plaque forming units (PFU) in a cell culture system for titration of FRV.

2. Materials

2.1 Equipment/instrumentation

- 2.1.1 2-ml self-refilling repetitive syringe¹
- 2.1.2 Micropipettor: 200 μ l²
- 2.1.3 Blender³
- 2.1.4 1000-ml borosilicate glass media bottle with screw-top lid⁴
- 2.1.5 36° \pm 2°C, 5% \pm 1% CO₂, high-humidity incubator⁵ meeting the requirements of the current version of GDOCSOP004
- 2.1.6 Water bath⁶
- 2.1.7 Vortex mixer⁷

2.2 Reagents/supplies

- 2.2.1 FRV Reference Virus⁸
- 2.2.2 Crandell feline kidney⁹ (CRFK) cell culture, free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR)

¹ Wheaton 13-689-50C, Fisher Scientific, Inc., 2000 Park Ln., Pittsburg, PA 15275 or equivalent

² Pipetman, Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent

³ Waring blender, Cat. No. 14-509-35, Fisher Scientific, Inc. or equivalent

⁴ Wheaton 219760, Fisher Scientific, Inc. or equivalent

⁵ Model 3158, Forma Scientific, Inc., Box 649, Marietta, OH 45750-0649 or equivalent

⁶ Cat. No. 15-461-10, Fisher Scientific, Inc. or equivalent

⁷ Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 700 Orville Dr., Bohemia, NY 11716 or equivalent

⁸ Reference quantities available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent

⁹ CCL-94, American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852-1776

Supplemental Assay Method for Titration of Feline Rhinotracheitis Virus
in Cell Culture

2.2.3 4-well tissue culture plates¹⁰

2.2.4 Minimum Essential Medium (MEM)

2.2.4.1 9.61 g MEM with Earle's salts without bicarbonate¹¹

2.2.4.2 2.2 g sodium bicarbonate (NaHCO₃)¹²

2.2.4.3 Q.S. to 1000 ml with deionized water (DW), and adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).¹³

2.2.4.4 Sterilize through 0.22-µm filter.¹⁴

2.2.4.5 Aseptically add:

1. 100 units/ml penicillin¹⁵
2. 50 µg/ml gentamicin sulfate¹⁶
3. 100 µg/ml streptomycin¹⁷
4. 5 ml lactalbumin hydrolysate or edamin¹⁸

2.2.4.6 Store at 4° ± 2°C.

2.2.5 Growth Medium

2.2.5.1 900 ml of MEM

2.2.5.2 Aseptically add:

1. 100 ml fetal bovine serum (FBS), heat inactivated at 56° ± 2°C for 30 ± 5 min
2. 10 ml L-glutamine¹⁹

¹⁰Cat. No. 7603705, ICN Biochemicals, Inc., 3300 Hyland Ave., Costa Mesa, CA 92626 or equivalent

¹¹Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgeman Ct., Gaithersburg, MD 20884 or equivalent

¹²Cat. No. S-5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent

¹³Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent

¹⁴Disposable filter, Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent

¹⁵Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent

¹⁶Gentocin solution, Cat. No. 0061-0464-04, Schering Laboratories or equivalent

¹⁷Cat. No. S-9137, Sigma Chemical Co. or equivalent

¹⁸Edamin S, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent

¹⁹L-glutamine-200 mM (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc. or equivalent

Supplemental Assay Method for Titration of Feline Rhinotracheitis Virus
in Cell Culture

2.2.5.3 Store at $4^{\circ} \pm 2^{\circ}\text{C}$.

2.2.6 2X Medium

2.2.6.1 100 ml 10X MEM²⁰

2.2.6.2 2.6 g NaHCO_3

2.2.6.3 340 ml DW

2.2.6.4 Sterilize through 0.22- μm filter.

2.2.6.5 Aseptically add:

1. 5 ml lactalbumin hydrolysate or edamin
2. 100 units/ml penicillin
3. 50 $\mu\text{g}/\text{ml}$ gentamicin sulfate
4. 100 $\mu\text{g}/\text{ml}$ streptomycin
5. 50 ml FBS, heat-inactivated

2.2.6.6 Store at $4^{\circ} \pm 2^{\circ}\text{C}$.

2.2.7 2% Tragacanth Gum (Trag)

2.2.7.1 20 g Trag²¹

2.2.7.2 1000 ml DW

2.2.7.3 Mix vigorously, small amounts at a time, with a blender set on high.

2.2.7.4 Pour 500 ml each into 1000-ml media bottles.

2.2.7.5 Sterilize by autoclaving at 15 psi for 35 ± 5 min.

2.2.7.6 Store at $4^{\circ} \pm 2^{\circ}\text{C}$.

2.2.8 Overlay Medium

2.2.8.1 Mix equal volumes of 2X Medium and 2% Trag.

²⁰Cat. No. 11435, Life Technologies, Inc. or equivalent

²¹Acros AC42138-5000, Fisher Scientific, Inc. or equivalent

Supplemental Assay Method for Titration of Feline Rhinotracheitis Virus
in Cell Culture

2.2.8.2 Store at $4^{\circ} \pm 2^{\circ}\text{C}$.

2.2.9 70% Ethyl Alcohol

2.2.9.1 73 ml ethyl alcohol²²

2.2.9.2 27 ml DW

2.2.9.3 Store at room temperature (RT)
($23^{\circ} \pm 2^{\circ}\text{C}$).

2.2.10 Crystal Violet Stain

2.2.10.1 7.5 g crystal violet²³

2.2.10.2 50 ml 70% Ethyl Alcohol

2.2.10.3 250 ml formaldehyde²⁴

2.2.10.4 Dissolve crystal violet in alcohol, add
remaining ingredients.

2.2.10.5 Q.S. to 1000 ml with DW.

2.2.10.6 Filter through filter paper.²⁵

2.2.10.7 Store at RT.

2.2.11 12 x 75-mm polystyrene tubes²⁶

2.2.12 25-ml pipette²⁷

2.2.13 3-ml syringe²⁸ and 20-ga x 1½-in needle²⁹

²²Denatured, 190 proof, Cat. No. 7018, J.T. Baker, Inc. or equivalent

²³Cat. No. C 0775, Sigma Chemical Co. or equivalent

²⁴37% by weight, Cat. No. F79, Fisher Scientific, Inc. or equivalent

²⁵Whatman #1, Cat. No. 1001, Fisher Scientific, Inc. or equivalent

²⁶Falcon 2058, Becton Dickinson Labware, 1 Becton Dr., Franklin Lakes, NJ 07417 or equivalent

²⁷Cat. No. 13-675-30, Fisher Scientific, Inc. or equivalent

²⁸Leur-Lok®, Cat. No. 309585, Becton Dickinson Labware or equivalent

²⁹Cat. No. 250107, Becton Dickinson Labware or equivalent

Supplemental Assay Method for Titration of Feline Rhinotracheitis Virus
in Cell Culture

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training in the preparation and maintenance of cell culture as well as in the propagation and maintenance of animal viruses.

3.2 Preparation of equipment/instrumentation

3.2.1 Set the water bath at $36^{\circ} \pm 2^{\circ}\text{C}$.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of CRFK Plates

3.3.1.1 Multiple 4-well plates are seeded with CRFK cells, in Growth Medium, at a cell count that will produce a monolayer after 48 ± 6 hr of incubation at $36^{\circ} \pm 2^{\circ}\text{C}$ in a CO_2 incubator. Cells older than 72 hr cannot be used in the test. Growth Medium is changed if excess acidity of the medium is observed or cells are not confluent 48 hr after seeding.

3.3.2 Preparation of Reference Virus

3.3.2.1 Rapidly thaw a vial of FRV Reference Virus in a $36^{\circ} \pm 2^{\circ}\text{C}$ water bath.

3.3.2.2 Dispense 1.8 ml of MEM into each of 8, 12 x 75-mm polystyrene tubes labeled 10^{-1} through 10^{-8} using a 2-ml repetitive syringe.

3.3.2.3 Transfer 200 μl of the Reference Virus to the tube labeled 10^{-1} , mix with the vortex mixer. Discard pipette tip.

3.3.2.4 Transfer 200 μl from the 10^{-1} labeled tube to the 10^{-2} tube, mix with the vortex mixer. Discard pipette tip.

3.3.2.5 Repeat Step **3.3.2.4** for each of the subsequent dilutions transferring 200 μl from the previous tube to the next dilution tube.

Supplemental Assay Method for Titration of Feline Rhinotracheitis Virus
in Cell Culture

3.3.3 Preparation of Trag

3.3.3.1 Warm the Trag in $36^{\circ} \pm 2^{\circ}\text{C}$ water bath for 60 ± 10 min prior to Step 4.6. Approximately 35 ml per plate is required.

3.4 Preparation of the sample

3.4.1 Rehydrate a vial of the Test Vaccine according to the manufacturer's instructions using a syringe and needle. Allow to incubate for 15 ± 5 min at RT.

3.4.2 Dispense 1.8 ml MEM into each of 6, 12 x 75-mm polystyrene tubes labeled 10^{-1} through 10^{-6} using a 2-ml repetitive syringe.

3.4.3 Transfer 200 μl from the Test Vaccine to the tube labeled 10^{-1} , mix with the vortex mixer. Discard pipette tip.

3.4.4 Transfer 200 μl of the tube labeled 10^{-1} to the tube labeled 10^{-2} , mix with the vortex mixer. Discard pipette tip.

3.4.5 Repeat Step 3.4.4 for each subsequent dilution through 10^{-6} transferring 200 μl from the previous tube to the next dilution tube.

4. Performance of the test

4.1 Decant the Growth Medium from the CRFK Plates.

4.2 Inoculate 1 well/dilution with 200 μl /well from dilutions 10^{-6} through 10^{-3} of the Test Vaccine into CRFK Plates. **Note:** The same pipette tip may be used if starting with the most dilute dilution. Gently rotate the plates to evenly disperse the inoculum.

4.3 Inoculate 1 well/dilution with 200 μl /well of the dilutions 10^{-8} through 10^{-6} of the Reference Virus. Gently rotate the plates to evenly disperse the inoculum.

4.4 One uninoculated well serves as a negative cell control.

Supplemental Assay Method for Titration of Feline Rhinotracheitis Virus
in Cell Culture

4.5 Incubate the inoculated CRFK Plates at $36^{\circ} \pm 2^{\circ}\text{C}$ for 75 ± 15 min in a CO_2 incubator.

4.6 Add 8 ml/well of the Overlay Medium (see section **2.2.8**) to the plates with a 25-ml pipette. Discard any unused, warmed Overlay Medium.

4.7 Incubate the CRFK Plates undisturbed at $36^{\circ} \pm 2^{\circ}\text{C}$ for 96 ± 12 hr in a CO_2 incubator.

4.8 At the end of incubation, without removing the Overlay Medium, pipette 5 ml of the Crystal Violet Stain (see section **2.2.11**) into each well of the plates with a 25-ml pipette.

4.9 Allow plates to incubate at RT 25 ± 5 min.

4.10 Wash the Overlay Medium and the Crystal Violet Stain from the cell monolayers by dipping each plate several times in a container with running water from the cold water tap. Allow to air dry.

4.11 PFU counting. If FRV and feline calicivirus (FCV) plaques are counted together in a combination vaccine, the FRV plaques will contrast markedly from FCV. The FCV plaques are larger (averaging 3 mm to 4 mm in diameter), with a fuzzy edge.

4.11.1 The FRV PFU are visible as small, approximately 1 mm diameter, clear, circular areas with distinct edges in the cell monolayer where the cells have been destroyed by the virus.

4.11.2 Count the number of FRV PFU for each well.

**Supplemental Assay Method for Titration of Feline Rhinotracheitis Virus
in Cell Culture**

4.12 The titer is expressed as PFU per dose of vaccine and is calculated as follows, using the plaque count from the dilution well which contains 10-100 PFU:

Example:

Log ₁₀ of PFU (65)	= 1.8
Log ₁₀ of reciprocal of dilution counted (10 ⁻³)	= 3.0
Log ₁₀ of reciprocal of dose factor	
$\frac{200 \text{ } \mu\text{l inoculum}}{1 \text{ ml-dose}} = \frac{1}{5}$	
	= 0.7
Total	= 5.5

Titer of the vaccine is 10^{5.5} PFU per ml dose.

5. Interpretation of the test results

5.1 For a valid assay, the calculated titer of the FRV Reference Virus must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.2 The uninoculated cell controls cannot exhibit any plaques, cytopathic effects, or cloudy media that would indicate any contamination.

5.3 For a satisfactory result, the titer of the Test Vaccine must be equal to or greater than the titer specified in the Outline of Production.

5.4 If the titer is less than the titer specified in the Outline of Production, then the Test Vaccine may be retested as stated in 9 CFR 113.8(b)(2).

6. Report of test results

6.1 Report test results as PFU/dose.

6.2 Record all test results on the test record.

Supplemental Assay Method for Titration of Feline Rhinotracheitis Virus
in Cell Culture

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.315.

7.2 Cottral GE: 1978, Manual of standardized methods for veterinary microbiology. Comstock Publishing Associates, p. 731. Ithaca, NY.

8. Changes

8.1 This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.